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Final Summary Technical Report:

US Department of Energy – Office of FreedomCar and Vehicle Technologies and US Centers for Disease Control and Prevention - National Institute for Occupational Safety and Health Inter-Agency Agreement research on "The analysis of genotoxic activities of exhaust emissions from mobile natural gas, diesel, and spark-ignition engines".

Overview:

The US Department of Energy – Office of Heavy Vehicle Technologies (now the DOE-Office of FreedomCar and Vehicle Technologies) signed an Interagency Agreement (IAA) with NIOSH, #01-15 DOE, 9/4/01, for "The analysis of genotoxic activities of exhaust emissions from mobile natural gas, diesel, and spark-ignition engines"; subsequently modified on 3/27/02 (DOE IAG#01-15-02M1); subsequently modified 9/02/03 (IAA Mod # 01-15-03M1), as "The analysis of genotoxic activities of exhaust emissions from mobile internal combustion engines: identification of engine design and operational parameters controlling exhaust genotoxicity". The DOE Award/Contract number was DE-AI26-01CH11089. The IAA ended 9/30/06.

This is the final summary technical report of National Institute for Occupational Safety and Health research performed with the US Department of Energy – Office of FreedomCar and Vehicle Technologies under that IAA: (A) NIOSH participation was requested by the DOE to provide in vitro genotoxicity assays of the organic solvent extracts of exhaust emissions from a suite of in-use diesel or spark-ignition vehicles; (B) research also was directed to develop and apply genotoxicity assays to the particulate phase of diesel exhaust, exploiting the NIOSH finding of genotoxicity expression by diesel exhaust particulate matter dispersed into the primary components of the surfactant coating the surface of the deep lung; (C) from the surfactant-dispersed DPM genotoxicity findings, the need for direct collection of DPM aerosols into surfactant for bioassay was recognized, and design and developmental testing of such samplers was initiated.

DISCLAIMER

(A) Bacterial mutagenicity assays and mammalian cell in vitro genotoxicity assays for chromosomal or DNA damage were performed on the organic solvent extracts of exhaust particulate matter (PM) and semi-volatile organic compounds (SVOC) collected from a random set of spark-ignition or diesel engine vehicles at the Southwest Research Institute (SwRI) in the DOE study; the engines were run on a standardized engine loading and speed driving cycle on a dynamometer at SwRI; and chemical and non-genotoxicity bioassays were performed on the exhaust materials by other DOE-affiliated laboratories (Desert Research Lab; Lovelace Respiratory Research Institute), referenced in [1] and [2]. The NIOSH genotoxicity studies were performed upon organic solvent extracts: of a combined diesel vehicle exhaust PM sample; a combined spark-ignition engine vehicle exhaust PM sample; and the matched pair of SVOC samples. NIOSH performed in vitro bioassays for gene mutation in bacteria, chromosomal damage as micronucleus (MN) induction in mammalian cells, and DNA damage by the single cell gel electrophoresis method in mammalian cells.

For these particular vehicles, the solvent extracts of the exhaust materials on a per unit mass basis were more active for the spark-ignition vehicles than for the diesel vehicles. However, on a fuel consumption basis, the diesel exhaust activity was greater. [3,4]. A caveat to the interpretation of these results is the is the differences in age and mileage of these particular vehicles. [2]. This NIOSH laboratory also has collaborated with other US Department of Energy research on bacterial mutagenicity studies of the organic solvent extract of filter-collected DPM, but from an industrial-scale stationary diesel engine operated on standard diesel fuel DF2, and on a Fischer-Tropsch synthesis fuel, mapping broad changes in bacterial mutagenic activity versus engine speed and loading. Steady-state conditions. [McMillian MH, Cui M, Gautam M, Keane M, Ong T, Wallace W, and Robey E. Society of Automotive Engineers Technical Paper 2002-01-1699, pp. 1-18 , 2002].

(B) In addition to these studies of the organic solvent extracts of diesel exhaust components, DOE-NIOSH IAA research measured in vitro genotoxic activities of whole, non-solvent-extracted DPM, using a NIOSH research method for in vitro genotoxicity assay of DPM dispersed into a principal component of the surfactant that coats the airway surface of the deep lung.[5,6,7].

NIOSH research has found that when DPM is mixed into an aqueous dispersion of a phospholipid component o lung surfactant, the material coating the air-interface of the pulmonary alveoli and respiratory bronchioles, then the particles are able to express their genotoxic activities in vitro. That is, the diesel soot particles are not dissolved, but are active as surfactant coated and dispersed particles. This provides a method to retain particle structure and its effects on the expression of toxicities, modeling the conditioning and activity of respired particles depositing on the lung deep airways. The soot particles adsorb surfactant onto their surfaces from aqueous dispersion, making the so-conditioned particle surfaces hydrophilic and permitting the soot particles to disperse aqueous media.

Bacterial mutagenicity assays and mammalian cell mutagenicity, clastogenicity, and DNA-damage assays have been performed for DPM supplied by the Lovelace Respiratory Research Institute from the automotive diesel engine system used in DOE in vivo inhalation exposure animal model studies of diesel exhaust-induced lung tumors in rodents [5,6], and from National Institutes of Standards and Technology (NIST) standard reference material diesel soot SRM 2975 [7]. Both DPM expressed bacterial mutagenicity and mammalian cell clastogenic or DNA-damage activity for both solvent extract and surfactant dispersion preparations.

The findings of the studies of surfactant-dispersed DPM in vitro genotoxicity may be summarized: Whole non-extracted diesel exhaust particulate can express in vitro genotoxic activities when dispersed into a model lung surfactant. That activity of whole surfactant-dispersed ultrafine diesel exhaust particles can be comparable to the activity of their extracted organics in bacterial mutagenicity assay. In some cases, the whole DPM dispersed in surfactant expresses much greater chromosomal or DNA damaging activity that expresses by the organics extracted from an equal mass of DPM. That is, genotoxic activity can be exacerbated when the genotoxic compounds are associated with otherwise inert carrier ultrafine particles.

(C) From the results of these surfactant-mediated in vitro DPM genotoxicity studies, research was begun under this IAA to develop an aerosol sampler to collect respirable DPM from the exhaust or the atmosphere directly into surfactant. This is to permit bioassay of the exhaust materials so as to retain their ultrafine nano-structure and to avoid anomalous physical or chemical conditioning of the particles during collection. A concept was developed for a system for direct sampling DPM into surfactant [8], and a preliminary quantitative test of a method was begun under this IAA. This involves the collaboration of NIOSH, US Dept. of Energy, West Virginia University, and University of Southern California researchers. This is being pursued under a new DOE-FCVT – NIOSH IAA begun in late FY06: US Department of Energy – NIOSH Interagency Agreement (IAA) #06-08: DE-A126-06NT42821: "Novel collection and toxicological analysis techniques for IC engine exhaust particulate matter".

The DOE-NIOSH IAA work is summarized in limited detail in the following report. Further details can be found in the available scientific literature generated in these studies which is referenced and listed at the end of this report. This consists of peer-reviewed technical journal publications, and juried scientific conference presentations for which the power-point proceedings are available electronically on the web. Additional questions can be addressed to the NIOSH Principal Investigator, William E Wallace, PhD, NIOSH, 1095 Willowdale Road, Morgantown, WV, 26505. Phone (304) 285-6096; wwallace@cdc.gov.

A. Comparative In vitro genotoxicity of Exhaust Emissions of In-Use Diesel and Gasoline Engine Vehicles Operated on a Unified Driving Cycle

As part of a US Department of Energy multi-institutional study of the exhaust toxicities of modern gasoline, diesel, and natural-gas mobile vehicle engines, NIOSH performed in vitro bioassays for gene mutation in bacteria, chromosomal damage as micronucleus (MN) induction in mammalian cells, and DNA damage by the single cell gel electrophoresis method in mammalian cells, on solvent extracts of diesel or gasoline engine exhaust particulate matter (PM) or vapor-phase semi-volatile organic compounds (SVOCs) for a set of vehicles in-use circa 2000 (Table 1). The was done in concert with chemical analyses [B. Zielinska, J. Sagebiel, J. McDonald, K. Whitney and D. Lawson, J. Air and Waste Mgt. Assoc., 2004, 54, 1138] and other toxicological analyses [J. C. Seagrave, J. D. McDonald, A. P. Gigliotti, K. J. Nikula, S. K. Selikop, M. Gurevich and J. L. Mauderly, Toxicol. Sci., 2002, 70, 212] of parallel samples of the same engine exhaust materials performed by other DOE –FCVT program contractors.

The study was designed to address in part the question of the *in vitro* genotoxic activities of exhaust emission materials from some typical recent-use spark-ignition gasoline engine and diesel engine light duty vehicles. It should be noted that mobile vehicle engines are in a state of rapid evolution and improvement in emissions reduction technology so the vehicles sampled in this study do not represent the best emission control capabilities now available. Diesel PM emission rates observed in this study are much higher than those which will be permitted under USEPA regulations being phased-in in 2007 for new diesel vehicles.

Acetone extracts of engine exhaust particulate matter (PM) and of vapor-phase semi-volatile organic compounds (SVOCs) collected from a set of 1998-2000 model year normal emitter diesel engine automobile or light trucks and from a set of 1982-1996 normal emitter gasoline engine automobiles or light trucks operated on the California Unified Driving Cycle were assayed at 72^0F or at 30^0F ambient temperature for *in vitro* genotoxic activities. Comparison measurements were made also on the extract of a standard diesel exhaust particulate material: National Institute of Standards and Technology (NIST) Standard Reference Material SRM1650a, typical of heavy-duty diesel engine particulate emissions of the early 1980s. Experimental design and results are briefly reviewed here. Details can be found in the references [1,3,4].

Table 1. Exhaust samples were collected from five gasoline engine vehicles and three diesel engine vehicles [a],

Vehicles	Year	Model	Odometer reading
Gasoline engine:			
	1982	Nissan Maxima	190,203
	1993	Mercury Sable	70,786
	1994	GMC 1500 pickup truck	68,325
	1995	Ford Explorer	76,733
	1996	Mazda Millenia	35,162
Diesel engine:			
	1998	Mercedes Benz E300	47,762
	1999	Dodge 2500 pickup truck	37,242
	2000	Volkswagen Beetle TDI	7,455

[a] Four pooled samples were prepared for assay: gasoline exhaust PM or SVOC; diesel exhaust PM or SVOC.

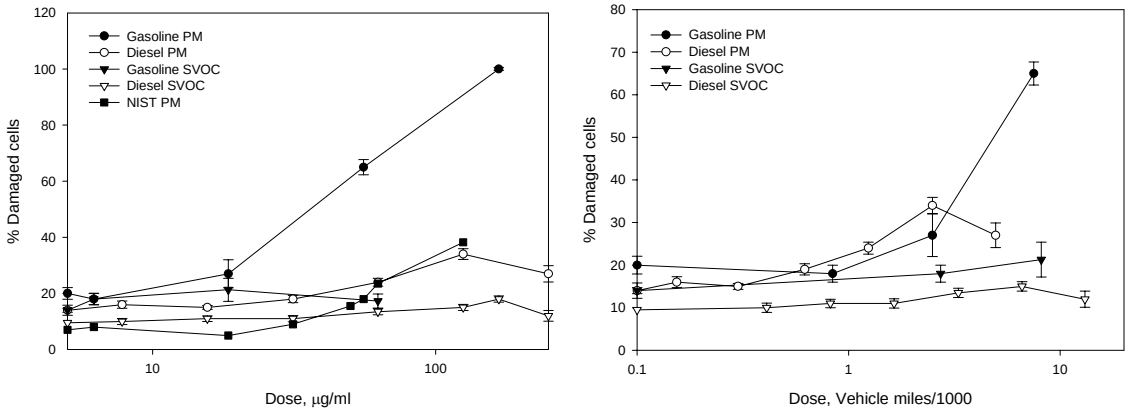
Table 2. Mutagenic activity per mass of extract and per vehicle mile for GP, DP, GSVOC, DSVOC, and NIST SRM 1650a, 72^0F ambient temperature.

Factor Combination	Group	Slope Estimate (Revertants/μg extract)	Slope Estimate (Revertants x 10^3/mile)
YG1024 - S9	GP (gasoline)	59.5[A]	440
	DP (diesel)	56.8[A]	2866
	G-SVOC	6.1	13.9
	D-SVOC	1.9	36
	NIST (SRM 1650a)	72.2 [A]	-
YG1024 + S9	GP	39.0 [B]	289
	DP	32.5 [C]	1640
	G-SVOC	4.9	11.2
	D-SVOC	1.4	26.6
	NIST	46.3 [BC]	-
YG1029 - S9	GP	19.4 [D]	144
	DP	13.1	661
	G-SVOC	5.7	13
	D-SVOC	1.0	19
	NIST	21.8 [D]	-
YG1029 + S9	GP	17.7	131
	DP	43.3 [E]	2185
	G-SVOC	5.2	11.9
	D-SVOC	1.8	34.2
	NIST	35.3 [E]	-

Table entries having the same superscript labels (A through E) are not significantly different ($p<0.05$)

Figure 1: Fraction of damaged cells from the SCGE assay vs. dose: (a) dose as μg extract/ml of culture medium; (b) dose as vehicle miles/ml of culture medium, 72^0F ambient temperature runs.

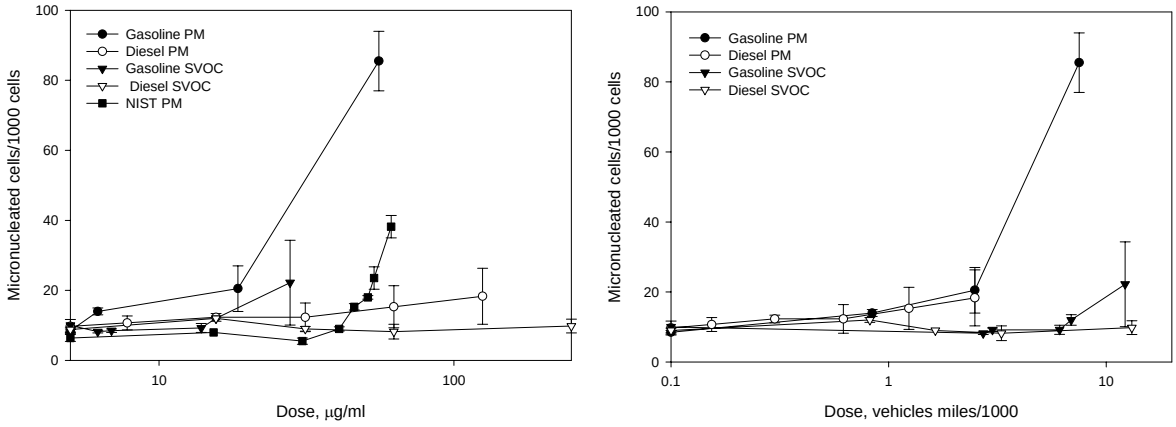
(a) (b)



Error bars are standard error of the means: N=8 for all gas & diesel PM and SVOC; N=12 for NIST. The abscissa scale is logarithmic. Values for the negative controls (zero dose) are shown on the ordinate.

Figure 2: Micronucleus induction expressed as micronucleated cells/1000 V79 cells vs. dose expressed as (a) μg extract/ml of culture medium or as (b) vehicle miles/ ml of culture medium, 72^0F ambient temperature runs.

(a) (b)

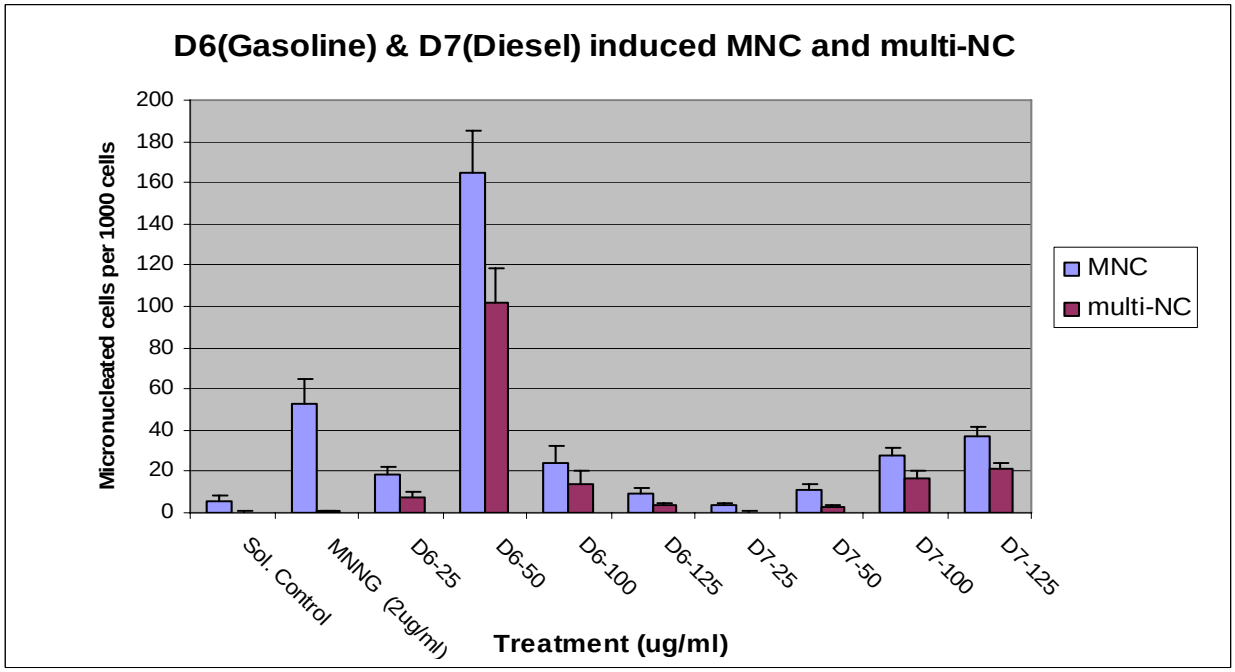


Error bars are standard error of the means: N ranging from 2 to 5. The abscissa scale is logarithmic. Values for the negative controls (zero dose) are shown on the ordinate.

Table 3. Mutagenic activity (revertants/ microgram of extract or kilo-revertants/vehicle mile) for D8 (Gasoline) or D9 (Diesel) particulate matter (PM) or semi-volatile organic compounds (SVOC) and NIST SRM 1650a, 30^0F ambient temperature.

	YG1024-S9		YG 1024+S9		YG1029-S9		YG1029+S9	
	Rev/ug	kR/mile	R/ug	kR/mile	R/ug	kR/mile	R/ug	kR/mile
D8 PM	33.8	574.6	39.5	671.5	14.7	250	30	510
D8 SVOC	5.6	37	2.5	16.5	6.8	44.9	2.8	18.5
D9 PM	90.1	14506	164	26404	29.5	4750	95	15295
D9 SVOC	7.1	339.7	9.2	440.2	8.5	406.7	13.8	660.3
NIST	54	NA	48.1	NA	22.3	NA	51.5	NA

Figure 3: Micronucleus induction expressed as micronucleated cells/1000 V79 cells vs. dose expressed as μg extract/ml of culture medium, 30^0F ambient temperature runs; (a) PM; (b) SVOC).



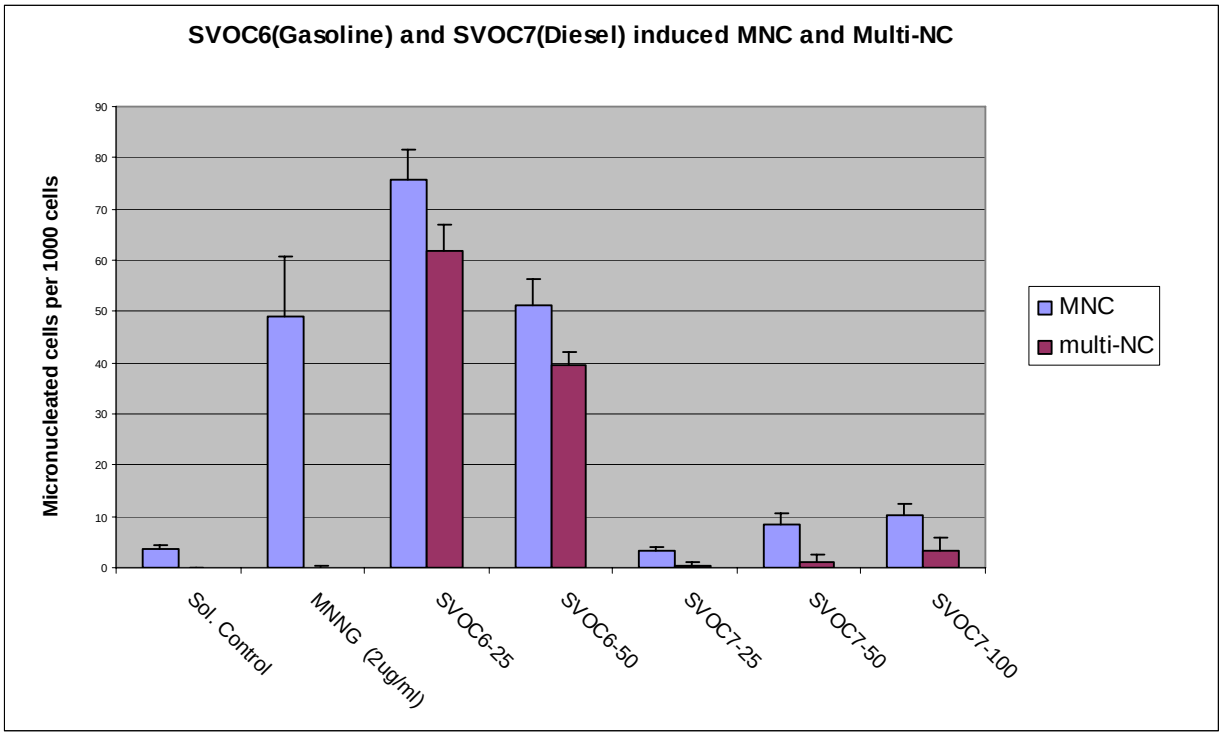
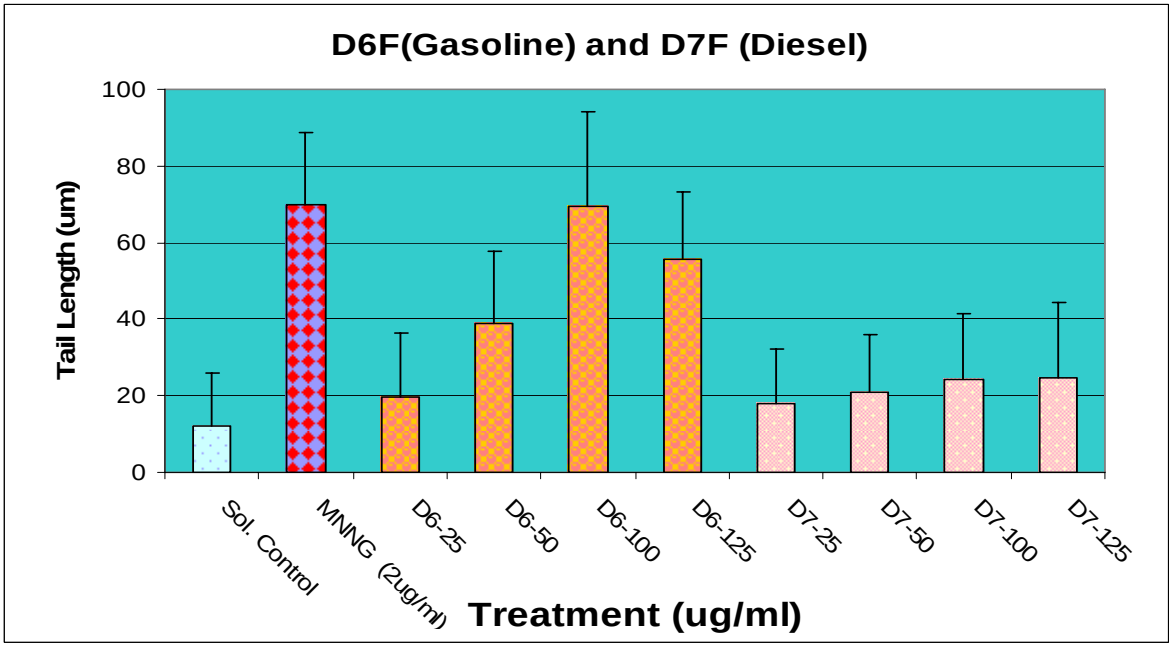


Figure 4: Fraction of DNA-damaged cells from SCGE assay vs. dose as µg extract/ml of culture medium; 30^0F ambient temperature runs; (a) PM; (b) SVOC).



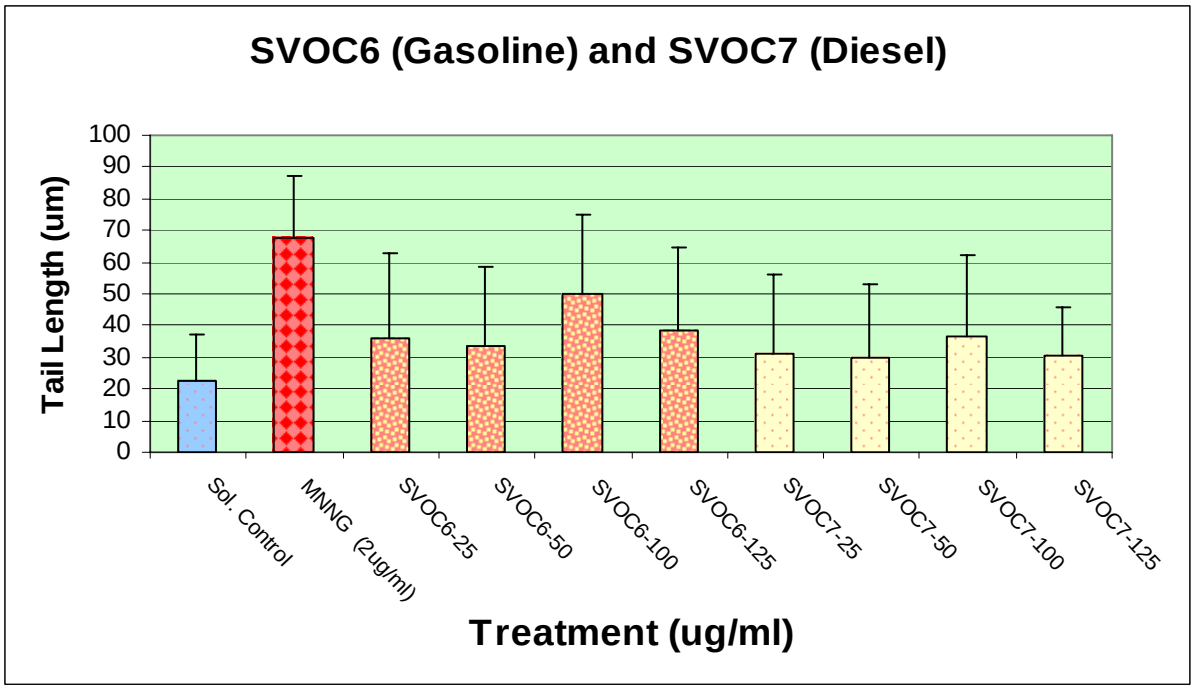


Table 4.

Qualitative Summary

72^0F operation

Sample	Gene mutation	DNA Damage	Chromosomal Damage
Diesel PM	+	(+) toxic	-
Gasoline PM	+	+	+
Diesel SVOC	weak	-	-
Gasoline SVOC	weak	(+) toxic	+

Qualitative Summary

30^0F operation

Sample	Gene mutation	DNA Damage	Chromosomal Damage
Diesel PM	+	weak	weak
Gasoline PM	+	+	+
Diesel SVOC	weak	weak	weak
Gasoline SVOC	weak	weak	+

All fractions of all materials, PM and SVOC, were highly mutagenic in the Salmonella reversion assays in both tester strains and conditions of the assay, with and without microsomal activation. Mutagenic activities expressed were similar on a mass basis for the gasoline and diesel engines particulate extracts; mutagenic activities of the NIST SRM particulate material extract, which represents older, 1980s diesel technology, were somewhat stronger. When normalized to doses based on vehicle miles, however, the mutagenicity versus dose slope estimates become much larger for the diesel exhaust, based on the greater engine exhaust emission production rate, often almost an order of magnitude greater for the diesels versus the spark-ignition engine emissions. The SVOC extracts in general were far less potent mutagens than were the PM extracts. The effect of the S9 microsomal fraction was complex, with the YG 1024 strain generally showing very similar or smaller dose-response mutagenicity slopes with the S9 addition, while the YG1029 strain did show significantly increased responses for the diesel particulate and SVOC extracts with microsomal fraction addition. The gasoline and diesel exhaust extracts both induced both frameshift and base-pair substitution mutations, with the diesel more active for the latter type. These results generally indicate that *in vitro* bacterial genotoxic activities of engine exhaust materials can be comparable on a mass of extract basis for spark-ignition gasoline engines and diesel engines, but were much stronger for these diesels on a mileage basis because of the much higher emission rates per mile for the diesel vehicles in this study.

Mammalian cell assays for DNA or chromosomal damage exhibited some qualitative differences between the diesel and gasoline extract samples distinct from the bacterial cell findings. DNA damage as measured by the SCGE "comet" assay was positive for gasoline engine exhaust PM, but diesel PM was comparatively weak on a mass basis, while comparable on a mileage basis, as shown in Fig. 1. Micronucleus induction in V79 cells did not parallel the SCGE results for all samples: the gasoline PM and gasoline SVOC extract activities for micronucleus induction were similar on a mass basis; this was the only case where SVOC activity approached that of the corresponding PM extract activity. Gasoline PM was much stronger than diesel PM extract for micronucleus induction on a mass basis, but was comparable on a mileage basis. However, diesel SVOC was inactive for micronucleus induction. The NIST sample was positive in this micronucleus assay, but with complex dose-response behavior, with activity initiating at the middle dose level. The results indicate that these gasoline engine exhaust materials were much stronger than the diesel exhaust materials on a mass basis for some mammalian cell genotoxic activities, with the gasoline exhaust activities comparable to or sometimes greater than the diesel exhaust activities on a mileage basis. This is not inconsistent with literature reports of some past studies of earlier generation vehicles, which saw significant mammalian cell genotoxic activities for gasoline engine exhaust extracts. One significant difference in the older studies was their use of leaded gasoline fuel, standard at the time. The NIOSH study suggested that leaded fuel use is not necessary for the production of gasoline exhaust extract genotoxic activity.

The chemical characterization work of Zielinski et al., 2004, on these materials found that the diesel exhaust particulate fraction contained much higher levels of nitro-polyaromatic hydrocarbon compounds (nitro-PAHs), which have been demonstrated to be a major contributor to the mutagenicity of diesel exhaust in the Salmonella mutagenicity assay. The detailed chemical analysis of the gasoline engine exhaust analytes showed generally lower concentrations of most relative to diesel exhaust, with the exception of PAHs, especially high molecular weight compounds such as indeno[cd]perylene, benzo[ghi]perylene, and coronene, which has also been

observed in earlier studies. This may at least partially explain why the micronucleus and SCGE assay results were consistently stronger for the gasoline exhaust PM, while the diesel PM was comparable on a mass basis in the Salmonella mutagenicity assay. Additional fractionation and preparation would be required before genotoxicity studies could test this hypothesis.

Genotoxic activities of exhaust products from these or any vehicles can be expected to change with the specific mode of operation. Under steady state operating conditions, diesel exhaust genotoxic activity can be strongly affected by conditions of operation, e.g., engine torque, rpm, and fuel injection timing [M. H. McMillian, M. Cui, M. Gautam, M. Keane, T. Ong, W. Wallace and E. Robey, Society of Automotive Engineers Technical Paper 2002-01-1699, pp. 1-18]. Detailing the effects on exhaust genotoxicant composition of these engine operational parameters, or of changing these parameters during vehicle operation as in acceleration, deceleration, or conditions of changing load, would require a different experimental design. Other factors that may affect the emissions include the fuel used and the effects of engine and exhaust aftertreatment system design; and vehicle age and maintenance can greatly affect emissions, e.g., “white smokers” and “black smokers”.

B. Analyzing Diesel Exhaust for Ultrafine Particle Structure and Composition Factors Affecting Toxicity.

Diesel exhaust control technology is rapidly evolving. Compared to the PM emission rates seen in the in-use vehicle study (above), significant reductions are mandated by the USEPA for 2007 to 2009 model years. While the new systems are being developed principally to meet the new EPA NOx and fine PM standards, there is the possibility that ultrafine (particle size below 0.1 micrometer) exhaust particles may be emitted from the new systems. There is a general concern in industry for new "nanoparticle" technologies: there is limited animal model evidence that some materials may have enhanced toxicities or present exacerbated health risk as ultrafine particles in contrast to their more limited toxic properties as respirable fine (micrometer-sized) particles. The above studies and essentially all in vitro assay results for diesel exhaust material reported in the scientific literature have been performed upon the organic solvent (e.g., acetone) extracts of filter-collected DPM. That is, the assays were performed upon chemicals dissolved out of the diesel exhaust particles; any effects of the particulate structure and complex composition of the particles are destroyed prior to the assay.

It had been suggested that the genotoxicant chemicals associated with DPM would not express their genooxtic activities if they were not extracted from the particles. And it was found that, unlike acetone or other organic solvents, the fluid coating the surface of the deep lung could not efficiently extract genotoxic chemicals from diesel soot particles. Thus the physiological significance of assays of materials solvent-extracted from diesel soot was questionable from the standpoint of representing the biological availability of the hydrophobic organic genotoxicants which would remain particle-bound in the lung.

That is, in vitro bioassays of organic solvent extracts of DPM appear not to model the biological availability in the lung of particle-bound organic genotoxicants. The in vivo physiological situation is this: upon depositing in the deep lung airspaces, particles first will encounter the thin alveolar hypophase, a liquid lining of the deep lung airspaces which is spread with pulmonary surfactants upon the air-liquid interface and which contains surfactants dispersed in micellar and lamellar forms within the liquid layer on the respiratory bronchioles and terminal alveoli. Studies have demonstrated that the primary components of lung surfactant do not extract materials with strong *in vitro* genotoxic activity from DPM. Research frequently uses a surrogate for lung surfactant consisting of a primary phospholipid component of lung surfactant, diacyl phosphatidylcholine, typically dipalmitoyl phosphatidylcholine (DPPC), dispersed into physiological salt-concentration saline. In addition to being a major chemical constituent of lung surfactant, DPPC as a surfactant layer at the water-air interface reproduces the principal surface tension altering effects of whole lung surfactant. The inability of a DPPC-in saline dispersion model of lung surfactant to extract organic genotoxicants DPM raised the question of whether the genotoxicants in insoluble hydrophobic DPM are able to express their activity under conditions of deposition in the lung. NIOSH research has found that mixing DPM into a DPPC-in-saline dispersion without filtering results in a surfactant dispersion of DPM that expresses *in vitro* genotoxic activity. That is, when diesel soot is mixed into the DPPC-in-saline surfactant, organic genotoxic chemicals are not extracted from the soot particles; but instead, the particles are coated with the surfactant. They thus acquire a hydrophilic surface, and they are solubilized,

not dissolved, into a dispersion in saline, retaining their ultrafine particle structure. The surface conditioning of the ultrafine particles that have adsorbed surfactant provides a plausible model of the surfactant surface-conditioning the particles would experience upon deposition in the lung. Furthermore, NIOSH research found that bacterial gene mutation activity expressed by the surfactant-dispersed (not extracted) DPM was quantitatively comparable to that expressed by organic solvent extracts of equal amounts of DPM, in assays of bacterial gene mutations. This addresses the question of how a hydrophobic particle-bound genotoxicant can be biologically available for genotoxic activity in the lung without dissolution; and it provides the basis for a bioassay protocol to determine ultrafine particle structural effects on the expression of genotoxic activity.

Surfactant-mediated in vitro genotoxicity testing of DPM was carried out to provide a capability for physiologically representative short-term in vitro genotoxicity testing of DPM while retaining ultrafine particle characteristics; and to develop a capability for collection of DPM directly into model lung surfactant in order to avoid possible chemical adulteration of DPM during sampling [5,6,7]. Such possible adulteration includes chemical modification of DPM resident on the collection filter during sampling by exhaust gases passing through the filter; and possible irreversible physical agglomeration of ultrafine particles during capture on the filter. In vitro genotoxicity assays for chromosomal aberrations (CA), micronucleus formation (MN), and gene mutation in V79 cells of solvent extract and of surfactant dispersion of an older automotive diesel engine soot were reported for a sample of DPM, graciously provided earlier by the Lovelace Respiratory Research Institute, that had been filter-collected from the dilution tunnel of a system used for chronic exposures of animals to whole diesel emissions. Exhaust was generated by 1980 Model General Motors 5.7 L V-8 engines operated on test stands over continuously repeating Federal Test Procedure urban duty cycles and burning certification D-2 Diesel Control Fuel.

For the preparation of surfactant dispersion, 100 mg DPPC was sonicated into 10 ml of 0.85% physiological sterile saline (PSS). For the DPM in DPPC dispersion, 25 mg of each DPM sample was mixed at 37°C into 2.5 ml of 10 mg DPPC/ml in PSS. For comparison solvent extracted samples, dimethyl sulfoxide (DMSO) dispersion of DPM was similarly prepared. Aliquots of these stock preparations were added to Eagles' Minimal Essential Medium to provide the DPM concentrations of 25 to 150 ug/ml in the cell systems.

After 72 h challenge of V79 mammalian-derived cells, chromosomal aberration were read on one hundred well-spread metaphases for each treatment, for chromatid gaps and breaks, isochromatid gaps and breaks, fragments, deletions, minutes, acentric ring, dicentromere and endoreduplications. The frequencies of CA's with and without gaps were compared to the solvent control (DPPC or DMSO) and to a positive control, N-methyl-N'-nitroso-N-nitrosoguanidine (MNNG), using a X^2 test. Treatment with DPM in DPPC dispersion in saline significantly increased the number of aberrant cells at concentrations of 25 to 150 *u*g/ml, with the effect generally increasing with concentration. (Table 5). A parallel elevation in the total number of CA was also observed. The difference between treated and control groups, both with and without gaps, was statistically significant.

Micronucleus induction in a similarly prepared V79 cell system was determined after 24-hr treatment. Micronucleated cells (MN) in treated and control cells were scored. A criterion for MN scoring was that their diameter must be no larger than one-third of that of the main nucleus. The frequency of MNC, expressed as the number of MNC cells per 1000 cells, was determined by counting 1000 cells on 6 separate slides for each treatment. DPM in DPPC induced MN formation activity that generally increased with concentration and was statistically significant at the highest concentration tested. DPM in DMSO significantly increased MN formation in V79 cells at all but the lowest concentration, with activity increasing with concentration (Table 6).

For assay of induction of 6-thioguanine-resistant forward mutation in eukaryotic cells, V79 cells were similarly prepared and incubated with DMSO or DPPC preparations of DPM for 24 h, and then after 7-day expression time, 6-TG/ml was added. Survival was measured after 10 days to estimate mutations. Table 7 gives the frequency of TG mutants obtained in the groups treated with DPM in DPPC-saline and also in DMSO. The results show that neither preparation significantly affected the frequency of TG mutants. This was confirmed in a repeated experiment. Data on relative survival, Table 5, indicate that DPM was toxic to V79 cells only at the highest concentration (150 μg/ml) tested.

These findings confirmed that genotoxic activity associated with diesel particles inhaled into the lung may be bioavailable by virtue of the dispersion properties of pulmonary surfactant components. Both CA and MN in vitro assays showed genotoxic responses for DPM dispersed in DPPC in saline, a primary component of pulmonary surfactant. CA analysis was more sensitive than the MN assay to the DPM. In vitro MN testing with mammalian cells such as V79 cells may provide a simple assay for screening the genotoxicity of diesel samples. Neither DPPC nor DMSO dispersion of DPM produced a mutagenic response in the in vitro TG gene mutation assay using V79 cells. This is consistent with literature reports of insensitivity of V79 cells to diesel soot in this assay.

Numerous conventional short-term in vitro assays of individual organic compounds have shown good sensitivity and specificity for correlation of assay results with in vivo tumorogenesis. Surfactant conditioning demonstrated can extended such in vitro bacterial and mammalian cell testing to insoluble NP materials. The NIOSH results indicate dispersion in phospholipid surfactant of 5 to 10 mg DPPC per square meter particulate material surface area can permit full expression genetic toxicity in mammalian cell assays. In vivo, pulmonary surfactant is well in excess of amounts needed to provide such adsorption and solubilization of respired NP. By calculation from literature values of the amount of lavageable lung surfactant and the half life time of surfactant replacement, lung surfactant is in quantity to solubilize some 100 times or more of the DPM that would be respired under occupational exposures at the ACGIH proposed exposure limit of 0.1 gm DPM per workday.

Table 5. Chromosomal Aberrations (CA) Induced by DPM in V79 Cells

Treatment	Conc. µg/ml	Aberrant Cells (%)	CA / 100 Metaphase [a]													Total CA [b]	
			Chromatid				lsochromatid									With Gaps	Without Gaps
			G	B	M	F	G	B	M	TD	AC	DC	IC	N	Other		
DPPC [a]	0.1	10	6	2	0	0	1	0	0	0	0	1	0	0	0	10	3
DPM [a] in DPPC	25	27[c]	11	0	0	0	3	0	3	5	0	5	0	1	0	28[c]	14[c]
DPM in DPPC	50	35[c]	12	6	0	0	8	0	1	6	0	2	1	1	1	38[c]	18[c]
DPM in DPPC	100	33[c]	15	3	1	6	9	0	1	2	1	3	1	0	1	43[c]	19[c]
DPM in DPPC	150	43 [c]	14	7	0	0	11	4	0	5	0	6	0	1	4	52[c]	27[c]
MNNG [a]	1	62 [c]	24	21	0	6	4	7	4	5	1	13	1	4	5	95[c]	67[c]

[a]Abbreviations: G, gaps; B, breaks; M, minutes; F, fragments; TD, terminal deletions; AC, accentric rings; DC, dicentric chromosomes; IC, interchanges; N, numerous aberrations; Other, including endoreduplication and polyploidy; DPPC, dipalmitoyl phosphatidylcholine; DPM, diesel particulate material; MNNG, N-methyl-N'-nitroso-N-nitrosoguanidine.

[b] 100 metaphases scored

[c] $p<0.05$ by χ^2 test

Table 6. Frequency of micronucleated cells (MNC) in V79 cells treated with DPM for 24 hours

Chemical	Concentration	Frequency of MNC[a]	Total number
Total Number	μg/ml	mean ±SD	of MNC[b]
DPPC[c]	5	5.7±0.82	34
DPM[c] in DPPC	25	6.5±1.05	39
DPM in DPPC	50	8.2±1.33	49
DPM in DPPC	100	6.8±1.47	41
DPM in DPPC	150	9.5±1.64	57[e]
DMSO[c]	5[d]	6.5±2.51	39
DPM in DMSO	25	5.2±1.60	31
DPM in DMSO	50	13.3±1.75	80[e]
DPM in DMSO	100	11.8±1.94	79[e]
DPM in DMSO	150	14.2±1.72	85[e]
MNNG[c]	1	48.0±5.55	288[e]

[a]MNC per 1000 cells; n=6.

[b]Based on 6000 cells scored in each of 2 experiments.

[c]Abbreviations: DPPC, dipalmitoyl phosphatidylcholine; DPM, diesel particulate material; DMSO, dimethyl sulfoxide; MNNG, N-methyl-N'-nitroso-N-nitrosoguanidine.

[d]Concentration in μ1/ml.

[e]Different from solvent control (DPPC or DMSO) at $p < 0.05$

Table 7. Results of 6-thioguanine-resistant gene mutation assay for DPM in V79 cells

Chemical	Concentration (μg/ml)	Relative Survival (%)	Mutants per 10^6 Survivors
DPPC[a]	5	100±9.5	6.1±1.3
DPM[a] in DPPC	25	174.9±29.2	6.6±2.4
DPM in DPPC	50	133.0±18.7	3.3±2.1
DPM in DPPC	100	105.2±13.1	10.8±3.1
DPM in DPPC	150	82.8±12.6	2.5±1.4
DMSO[a]	5 [b]	100±4.9	6.2±2.9
DPM in DMSO	25	38.8±13.4	6.1±1.9
DPM in DMSO	50	115.1±11.4	5.5±2.3
DPM in DMSO	100	99.4±7.9	9.1±5.6
DPM in DMSO	150	83.8±10.1	7.8±3.4
MNNG[a]	1	15.5±3.0	114.9±19.8

[a] See footnote c in Table 2 for abbreviations.

[b]Concentration in μl/m1

A concern for the extrapolation from in vitro genotoxic activity to possible disease hazard, including the case of surfactant-dispersed DPM, is that DPM induction of lung tumors in animal models has been under conditions of "particle overload" exposures rather than under conditions representative of occupational exposures. Conditions of in vitro cell challenge leading to induction of genotoxic activities have usually been at DPM-to-cell concentrations far exceeding doses conferred by one day of exposure at the ACGIH limit of 0.1 mg per workday. However, incomplete lung clearance or sequestration of DPM within pulmonary cells can lead to increasing lung loads of DPM with increasing exposure times. This has been reported in the literature for inhalation exposures of the rat to 0.25 mg to 6 mg DPM per cubic meter of air, which resulted in residual lung burdens after 7 to 112 days of 0.2 to 12 mg retained DPM. Such amounts would be modeled for in vitro study by concentrations in the range of 1 to 10 microgram DPM per square centimeter of plated cells, which are within concentrations used in these NIOSH-DOE in vitro mammalian cell studies of surfactant-dispersed DPM, which show clastogenic and DNA damage.

Further research on surfactant-dispersion and genotoxicity testing on DPM was begun under this IAA on a reference material from the US National Institute for Standards and Technology (NIST). This was to provide information on a "positive control" DPM for other research labs that might pursue surfactant-mediated diesel genotoxicity studies in support of diesel engine technology development. Preliminary results were peer-review selected for presentation at the 2006 DOE-sponsored Diesel Engine Efficiency and Emission Control Research Conference. The presentation materials are available on the web [7]. The NIST diesel soot was assayed for bacterial mutagenicity and mammalian cell DNA damage and chromosomal damage, as an acetone/dimethylsulfoxide extract and as a dispersion into DPPC/saline. For this particular soot, the mammalian cell assays of micronucleus induction and single cell gel electrophoresis measure of DNA single or double strand breaks, resulted in much higher expression of genotoxicity for the surfactant-dispersed particles in comparison to the activity of the extracted genotoxicants. That is, on a mass basis, genotoxic activity to mammalian cells was exacerbated for the case of the genotoxicants bound to ultrafine particles.

Bacterial mutagenicity assay of filter-collected NIST Diesel Exhaust Standard Material 2975 used the "micro-suspension" mode of the histidine reversion gene mutation assay in Salmonella typhimurium YG1024 or YG1029, with or without microsomal enzyme activation. Surfactant dispersion used DPPC ultrasonically dispersed in physiological sterile saline at a concentration of 2.5 mg DPPC / ml PSS. DPM was mixed into the dispersion at a concentration of 1 mg DPM/ 2.5 mg DPPC / 1 ml PSS. This stock dispersion then was diluted with PSS to provide samples of 13.3, 40, 120 microgram DPM in 10 micro-liter PSS. The solvent preparation was made by acetone extraction, evaporation, and dissolution into dimethylsulfoxide (DMSO) at 2 mg DPM extract / ml DMSO. Dilution with PSS provided samples of 13.3, 40, 120 microgram extract in 10 microliter PSS. The test protocol for the micro-suspension test was: mix samples of0.0133, 0.040, 0.120 mg DPM or solvent-extract of DPM in 0.01 ml PSS, add .065 ml PSS or S9 preparation, add .025 ml of YG1024 or YG1029 @ 1-2 x 108 cells/ml; preincubate the mixture 30 minutes at 37C; mix with 2.5 ml top agar containing .05 mM biotin + histidine; then grow the cells for 48 hr (YG1024) or 72 hr (YG1029); and then count the formed colonies.

Mammalian Cell clastogenic damage assay of NIST SRM 2975 DPM used the micronucleus assay in V79 cells at 2x106 cells / ml medium, grown 24h prior to challenge. The positive

control was MNNG. The surfactant was DPPC ultrasonically dispersed in physiological sterile saline to a dispersion concentration of 2.5 mg DPPC / ml PSS. The same DPM and DPPC stock dispersion concentration of 1 mg DPM/ 2.5 mg DPPC / 1 ml PSS was prepared, but diluted with PSS to samples of 40, 120,180 microgram DPM / ml PSS. The solvent preparation was to extract DPM with acetone, evaporate the extract, dissolve the extract residue in DMSO to 2 mg DPM extract / ml DMSO, and subsequently dilute this stock with PSS to provide samples of 200, 600, 900 microgram extract in 5 ml medium. The assay then used 24 h incubation of V79 cells in 5 ml medium with 40, 120, 180 microgram/ml of (X) solvent extract of DPM or (D) DPPC-dispersed DPM. After 24 hr challenge the cells were rinsed, 5 ml medium replaced; and incubated another 24 hr. cells then were harvested, fixed, stained and slides prepared for optical microscopy examination, scoring 6 x 1000 cells (n=6) for each concentration for each of 2 runs.

Mammalian cell DNA damage assay of the NIST 2975 soot used the single-cell gel electrophoresis assay for single- or double-strand DNA damage under the same V79 cell system assays preparations as the micronucleus induction assays. After 24 hr challenge then SCGE was performed , and 100 cells read for each treatment for each of two runs.

Figure 5 shows comparable activity for bacterial mutagenicity induction by a given mass of DPM dispersed in DPPC surfactant versus the activity of the solvent extract of the same mass of DPM. Figure 6.a shows micronucleus induction by a given mass of DPM dispersed in DPPC surfactant, but versus the activity of an equal mass of extract. Figure 6.b shows the data of Figure 6.a normalized to the amount of DPM from which the extract was taken; that is comparing the activity of a concentration of DPPC-dispersed DPM versus the activity of the (smaller) amount of dissolved organics that would be extracted from that same amount of soot. In the case of this particular NIST SRM2975 DPM, there were only 4% extractables. Figure 7 similarly shows the exacerbated activity for particle-borne genotoxicants in the SCGE DNA-damage assay. These results indicate that a synergistic affect on the expression of genotoxicity in mammalian cells can occur for organic genotoxicants when borne on respirable ultrafine carbonaceous soot.

In addition, a particulate carbon black sample containing no extractable organic genotoxicants, and the residual particulate material of the NIST 2975 DPM that survived acetone extraction were mixed into DPPC dispersion and assayed in the mammalian cell micronucleus assay. Neither was active. This was to test for a possible contribution to the genotoxic activity due to the ultrafine particulate matter per se, that is, due for genotoxic activity associated only with the ultrafine size of the particles. The data suggest that the presence of genotoxicants on the particles was necessary for the genotoxic activity; but that for this NIST sample the combination of genotoxicants on the ultrafine particle significantly exacerbated the genotoxic activity for mammalian cells, but not as strongly for bacterial mutagenic activity. Additional data is contained in the 2006 DEER Conference presentation [7]. Additional assays are being performed and the data is being analyzed toward a report for peer-reviewed journal submission, under the new US Department of Energy – NIOSH Interagency Agreement (IAA #06-08: DE-A126-06NT42821): “Novel collection and toxicological analysis techniques for IC engine exhaust particulate matter”, begun 4Q06.

C. Collecting DPM to Retain Ultrafine Particle Factors Affecting Toxicity

In vitro genotoxic activities assayed on organic solvent-extractable materials from DPM can vary greatly and systematically with diesel engine system mode of operation, fuel, and other engineering parameters. Further, the NIOSH studies of surfactant-dispersed DPM genotoxicity, show that genotoxic activities can be expressed by some DPM containing organic genotoxicants, as non-dissolved particulate dispersion in a principal component of lung surfactant; and that for some DPM the genotoxic activity for mammalian cells is quantitatively greater for the genotoxicants when they are ultrafine-particulate-borne, compared to their activities as solvent extracts.

Another consideration for filter collection and in vitro bioassay of DPM is the possibility of anoamlus conditioning of ultrafine DPM during filter collection by irreversible agglomeration of the ultrafine aerosol particles or chemical modification of the DPM held on the filter by lengthy exposure to heteroatomic comounds in the exhaust.

Collection of aerosolized DPM from the exhaust directly into surfactant dispersion for direct bioassay suggests a basis for sampling and testing ultrafine particulate exhaust materials with low mass of emissions vis a vis current emission standards, but with ultrafine particle size and associated high specific surface areas.

A first quantitative test of a sampling system for the collection of diesel exhaust ultrafine particulate material directly into DPPC/saline surrogate lung surfactant was been initited under this DOE-NIOSH IAA. This involved a collaborative effort by NIOSH, the US Department of Energy – Office of FreedomCar and Vehicle Technology, West Virginia University (WVU), and the University of Southern California (USC). Samples were collected from a commercial diesel engine operated on a dynamometer in the WVU diesel engine test lab, using a USC humidifier system to condition the exhaust stream to prepare ultrafine particles for efficient collection in a surfactant-laden impinger. Genotoxicity assays on the collected samples are underway and will be completed under the new DOE-NIOSH IAA. The concept is the subject of an Employee Invention Report filed with the US Centers for Disease Control, with joint DOE, NIOSH, West Virginia University, and University of Southern California authorship. Results will guide modification of the sampler and sampling protocol with subsequent additional testing and application in the new DOE-NIOSH IAA begun in late FY06.

Figure 5

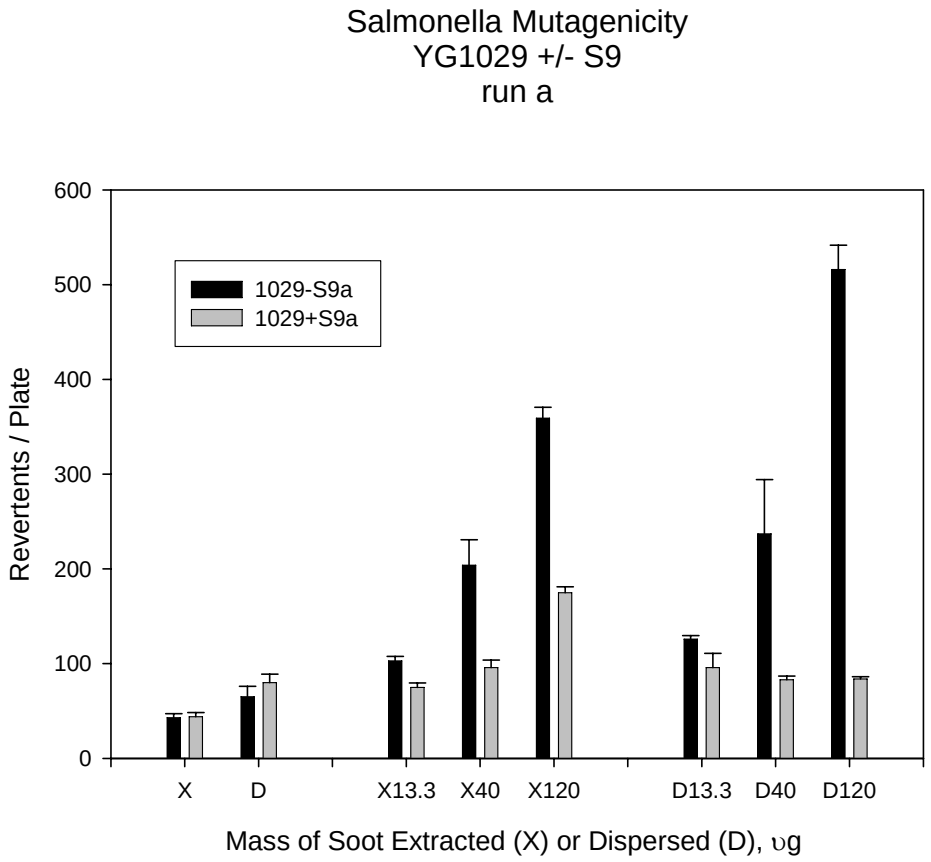
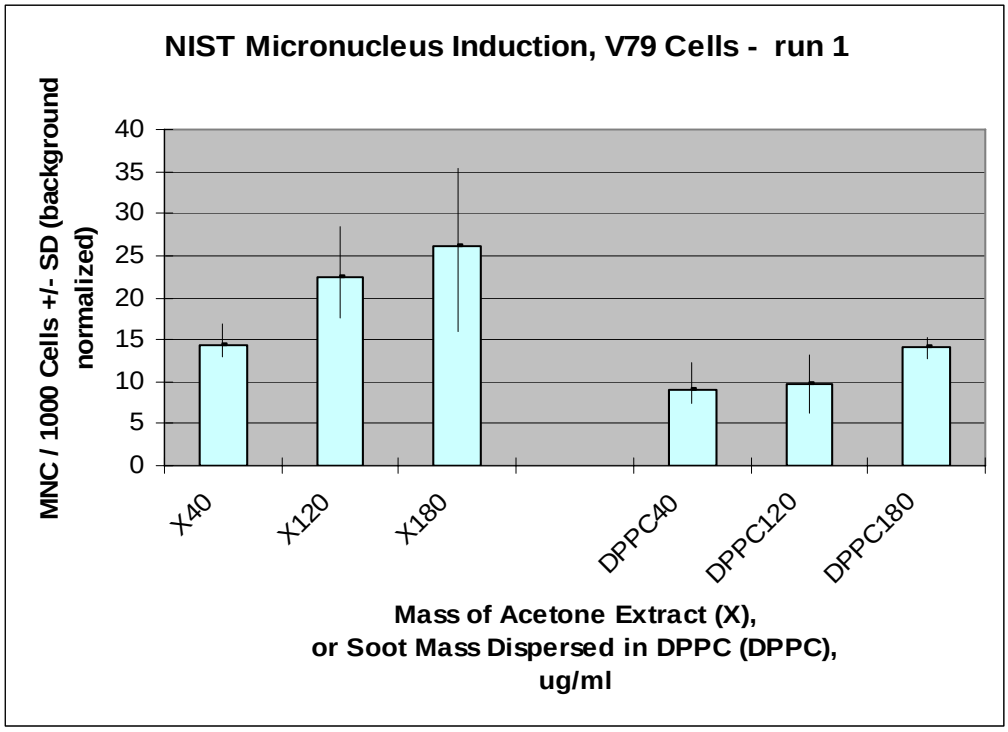

Salmonella Mutagenicity
YG1029 +/- S9
run a
1029-S9a
1029+S9a
Revertents / Plate
600
500
400
300
200
100
0
X
D
X13.3
X40
X120
D13.3
D40
D120
Mass of Soot Extracted (X) or Dispersed (D), υg

Figure 6


NIST Micronucleus Induction, V79 Cells - run 1
MNC / 1000 Cells +/- SD (background normalized)
40
35
30
25
20
15
10
5
0
X40
X120
X180
DPPC40
DPPC120
DPPC180
Mass of Acetone Extract (X),
or Soot Mass Dispersed in DPPC (DPPC),
ug/ml

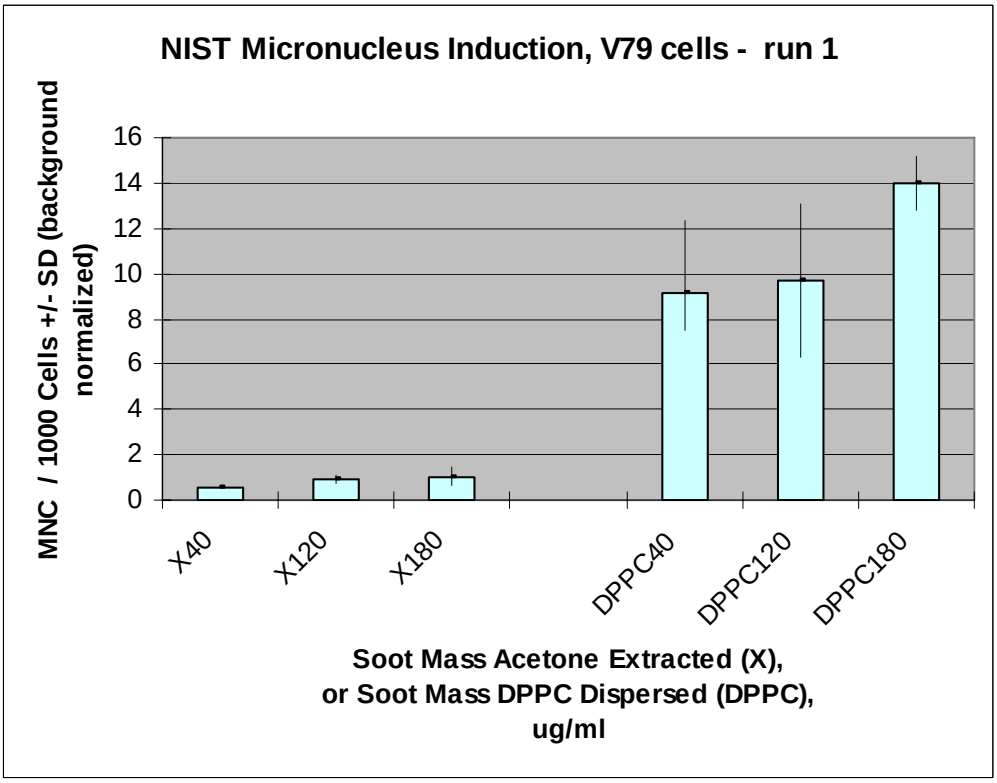
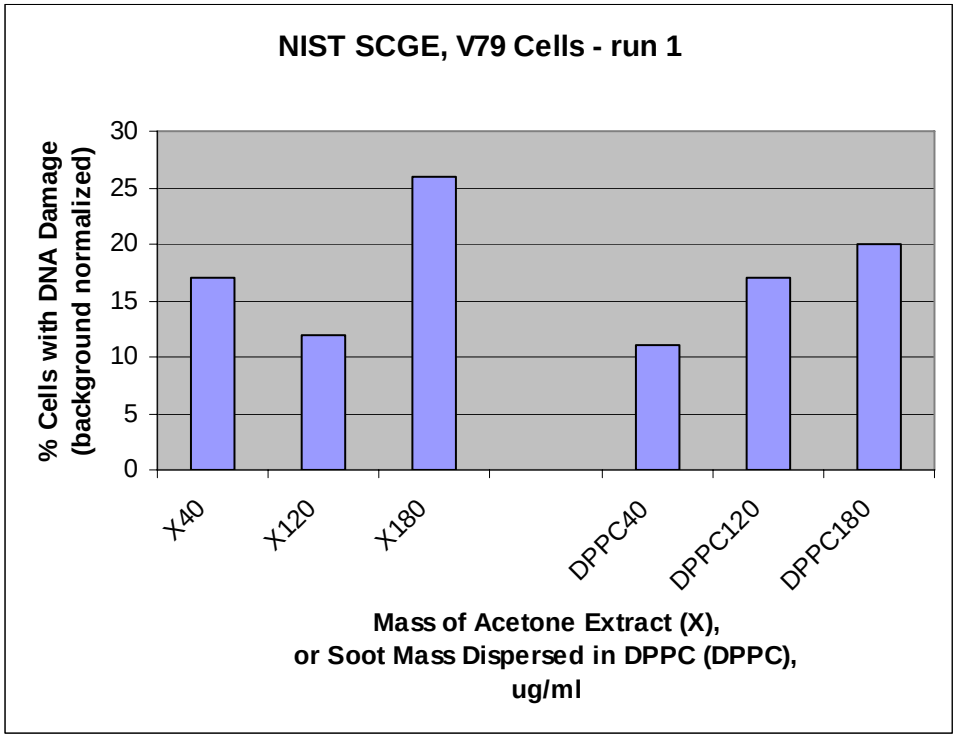
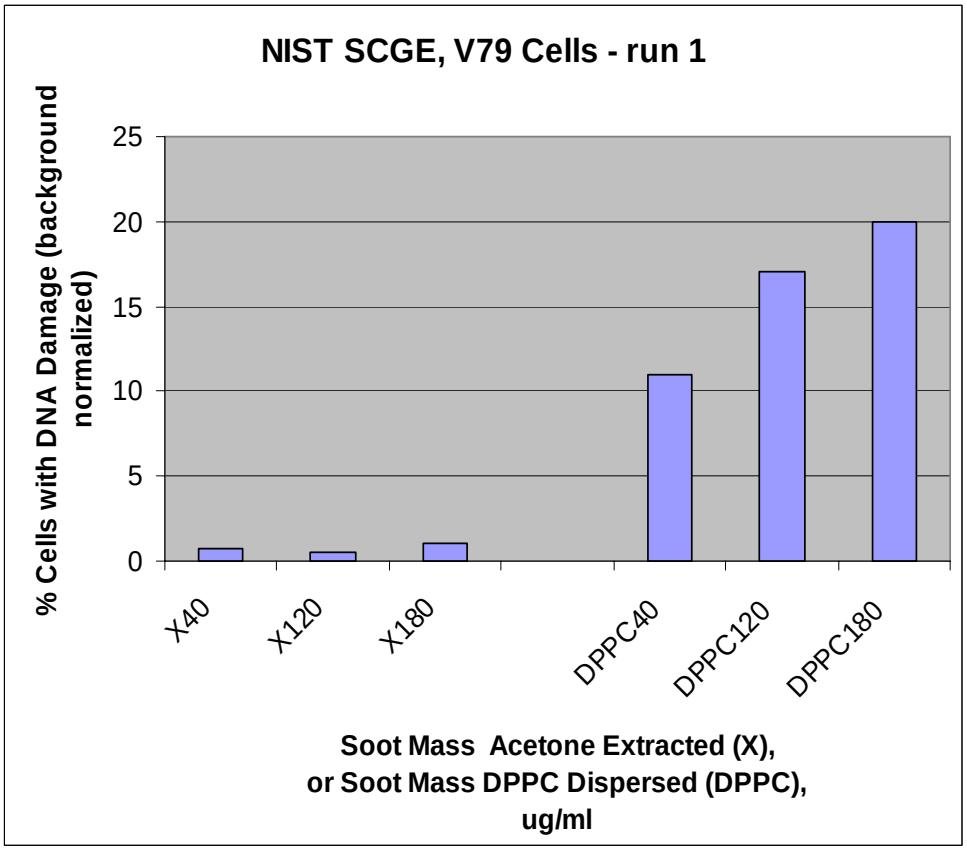

NIST Micronucleus Induction, V79 cells - run 1
MNC / 1000 Cells +/- SD (background normalized)
16
14
12
10
8
6
4
2
0
X40
X120
X180
DPPC40
DPPC120
DPPC180
Soot Mass Acetone Extracted (X),
or Soot Mass DPPC Dispersed (DPPC),
ug/ml

Figure 7


NIST SCGE, V79 Cells - run 1
% Cells with DNA Damage (background normalized)
30
25
20
15
10
5
0
X40
X120
X180
DPPC40
DPPC120
DPPC180
Mass of Acetone Extract (X), or Soot Mass Dispersed in DPPC (DPPC), ug/ml


NIST SCGE, V79 Cells - run 1
% Cells with DNA Damage (background normalized)
25
20
15
10
5
0
X40
X120
X180
DPPC40
DPPC120
DPPC180
Soot Mass Acetone Extracted (X), or Soot Mass DPPC Dispersed (DPPC), ug/ml

D. Research to Practice:

The US Environmental Protection Agency (EPA) is requiring more stringent controls of diesel vehicle exhaust emissions of respirable particulate matter and oxides of nitrogen; these are to be implemented over the next several years, beginning in 2007. US DOE and industry-supported research groups are mounting studies of prototype diesel engines with integrated emission control technologies toward meeting those exhaust emission control requirements. At the same time, there is concern with the possibility for production and release of ultrafine particulate exhaust materials from the new systems. While very low in mass of emissions, there is concern for ultrafine sized or "nano-particulate" matter in the final exhaust. DPM can have very small particle size, below 0.1 micrometer. That size itself or the associated high specific surface area of such ultrafine particles, that is, surface area per unit mass of ten to a hundred times that of typical fine-sized respirable particles, may present unknown respiratory or other health hazard. That is, there is expressed interest by government and industry for emissions of ultrafine particles from new technologies. Mass measures of particulate emissions may not be fully predictive of health consequences. In vitro genotoxic activity of the organic extracts of in diesel exhaust vary with engineering parameters.

Determination of the effects of engine design and operational parameters and new emission control technologies on exhaust PM genotoxic activity, measured so as to retain particulate phase properties and effects on genotoxicant bioavailability and activity, are needed in order to readily identify potential ultrafine particulate emissions with such heightened toxicity, and to inform diesel engine and exhaust control system development to control such emissions. The surfactant-mediated in vitro testing methods from these studies provide a basis for short-term genotoxicity assays modeling some insoluble-particle interactions in the lung that than can affect the in vitro expression of toxicity. Collection of diesel exhaust stream aerosol DPM directly into surfactant dispersion might permit retention of critical particulate properties significantly affecting the expression of DPM toxicities as they would occur upon deposition in the lung. Such collection and assay may be feasible, in time and effort, for on-going guidance of diesel emission control technology development, and to complement government – industry developmental testing of prototype diesel emission control systems to meet the new EPA diesel emission standards.

There now is general concern in industry and government for unexpected hazards that might be associated with exposures to ultrafine particles in the burgeoning field of "nano-particle" technology. Results of these DOE-NIOSH studies of ultrafine diesel exhaust particulate materials, have been solicited for consultation, presentation or review publication to inform toxicological investigations for emerging "nanoparticle" production or use [9,10,11,12], as well as for investigations of new diesel engine control technologies [13].

E. Reports:

1. Liu Y-Q, Keane M, Ensell M, Miller W, Kashon M, Ong T, Mauderly J, Lawson D, Gautam M, Zielinska B, Whitney K, Eberhardt J, Wallace WE. "In vitro genotoxicity of exhaust emissions of diesel and spark-ignition gasoline engine vehicles operated on a unified driving cycle". Journal of Environmental Monitoring 7: 60-66 (2005).

2. Mauderly J, Seagrave JC, Wallace W. "Office of Heavy Vehicle Technologies study of comparative toxicity of engine emissions. B. Status of toxicity results". [Abstract]. 7th Diesel Engine Emission Reduction (DEER) Conference; 2001; Proceedings Presentations: http://www.osti.gov/hvt/ . (2001).

3. Liu L, Keane M, Cui M, Ensell M, Miller W, Kashon M, Ong T, Wallace W. "In vitro genotoxicity of gasoline and diesel engine vehicle exhaust particulate and semi-volatile organic compound materials." 8th Diesel Engine Emissions Reduction (DEER) Conference; August 25-29, 2002. Proceedings Presentations: http://www.osti.gov/hvt/ . San Diego, CA. (2002).

4. Ensell M, Keane M, Ong T, Wallace W. "In vitro genotoxicity of particulate and semi-volatile organic compound exhaust materials from a set of gasoline and a set of diesel engine vehicles operated at 30^0 F". Ninth Diesel Engine Emissions Reduction (DEER) Conference; 2003. Proceedings Presentations: http://www.osti.gov/hvt/ (2003).

5. Gu Z-W, Keane MJ, Ong T, Wallace WE. "Diesel exhaust particulate matter dispersed in a phospholipid surfactant induces chromosomal aberrations and micronuclei but not 6-thioguanine-resistant gene mutation in V79 cells in vitro". J Toxicology & Environmental Health, Part A, Vol. 68(6) 431-444 (2005).

6. Shi XC, Harrison JC, Gu ZW, Keane MJ, Ong T, Murray DK, Wallace WE; "Diesel exhaust dispersion in a phospholipid lung surfactant for retention of nano-particulate structure in short-term bioassays"; 11th Diesel Engine Emission Reduction (DEER) Conference; 2005; Proceedings Presentations: http://www.osti.gov/hvt/ (2005).

7. Shi XC, Keane M, Ong T, Harrison J, Gautam M, Bugarski A, Wallace W; "In vitro mutagenic and DNA and chromosomal damage activity by surfactant dispersion or solvent extract of a reference diesel exhaust particulate material". Peer-review selected presentation. 12th Diesel Engine Efficiency and Emissions Reduction Conference. Detroit MI. Proceedings Presentation: http://www.cemamerica.com/doeevents/DEER/presentations.html (2006).

8. Wallace W, Keane M, Ong, T, Gautam M, Sioutas C, Eberhardt J, Bugarski A. "Respirable Particle Sampler for the Collection of Air-Borne Fine and Ultrafine Particles Directly Into Surrogate Lung Surfactants for Bioassay". Employee Invention Report, CDC-Office of Technology Transfer. (2006).

9. Wallace W, Keane M, Murray M, Chisholm W, Maynard A, Ong T. "Phospholipid Lung Surfactant and Nano-Particle Surface Toxicity: Lessons from Diesel Soots and Silicate Dusts". Invited submission; Peer review-accepted for publication: In press: Journal of Nanoparticle Research - Special Issue: Nanotechnology and Occupational Health. (2007).

F. Invited Presentations, Consultations

10. Nano-Toxicology Conference: “Pitfalls, Hurdles and Limitations of Research Design in Nanotoxicology”; Cambridge, Massachusetts. Invited presentation: “Artificial Surfactant Dispersion of NP for Toxicological Studies: Lessons and Concerns from Diesel Exhaust Particulate In Vitro Genotoxicity and Fine-sized Mineral/Metal Particle Toxicity and Disease Risk.”. 2006.

11. 2nd International Symposium on Nanotechnology and Occupational Health; Minneapolis, Minnesota. Invited presentation: “Phospholipid lung surfactant dispersion of NP for retention of size and structural properties in bioassay: lessons from diesel particulate material in vitro genotoxicity”. 2005.

12. National Toxicology Program, National Science Foundation, US EPA, US Airforce Office of Scientific Research: “National Nanotoxicology Workshop - Developing approaches for evaluation of toxicological interactions of nanoscale materials”. Invited participation. U. Florida, 2004.

13. Health Effects Institute (HEI) and Coordinating Research Council of the Engine Manufacturers Association - Advanced Collaborative Emissions Study (ACES). Invited participation: “Workshop on strategies to evaluate diesel emissions in the ACES Project”. Denver, CO. 2003.